Food Chemistry 211 (2016) 853–859

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A simple microplate-based method for the determination of α-amylase activity using the glucose assay kit (GOD method)

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ARTICLE INFO

Article history:
Received 20 February 2016
Received in revised form 11 May 2016
Accepted 13 May 2016
Available online 20 May 2016

Chemical compounds studied in this article:
Gallic acid (PubChem CID: 370)
(+)-Catechin hydrate (PubChem CID: 9064)
Rutin hydrate (PubChem CID: 45479757)
Maltose monohydrate (PubChem CID: 16211336)
Anhydrous dextrose (PubChem CID: 5793)
Sodium phosphate dibasic (PubChem CID: 24203)
Sodium phosphate monobasic (PubChem CID: 23672064)
Starch soluble (PubChem CID: 439341)
3,5-Dinitrosalicylic acid (PubChem CID: 11873)
Potassium sodium tartrate tetrahydrate (PubChem CID: 2724148)

Keywords:
Glucose oxidase
α-Amylase
Maltose
Amylase inhibitors
DNSA

ABSTRACT

For the first time, a reliable, simple, rapid and high-throughput analytical method for the detection and quantification of α-amylase inhibitory activity using the glucose assay kit was developed. The new method facilitates rapid screening of a large number of samples, reduces labor, time and reagents and is also suitable for kinetic studies. This method is based on the reaction of maltose with glucose oxidase (GOD) and the development of a red quinone. The test is done in microtitre plates with a total volume of 260 μL and an assay time of 40 min including the pre-incubation steps. The new method is tested for linearity, sensitivity, precision, reproducibility and applicability. The new method is also compared with the most commonly used 3,5-dinitrosalicylic acid (DNSA) method for determining α-amylase activity.

1. Introduction

Starch is a major component in our diet that is involved in energy production in the body. Main enzymes involved in the digestion of starch are salivary and pancreatic α-amylases and the small intestinal brush border α-glucosidases (Liu, Song, Wang, & Huang, 2011). Alpha amylase is an endoamylase that catalyze the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of internal α-D-(1-4) glycosidic bonds resulting in α-anomeric products (de Sales, de Souza, Simeoni, Magalhães, & Silveira, 2012; Liu et al., 2011; Sivaramakrishnan, Gangadharan, Nampoothiri, Soccol, & Pandey, 2006). It should be noted that neither terminal glucose residues nor α-D-(1-6) linkages can be cleaved by α-amylase. Therefore, the resulting products of α-amylase action are the dextrins and oligosaccharides (de Sales et al., 2012).

Other than being the main energy source, excess intake of starchy food is associated with the development of type 2 diabetes mellitus (T2DM) (Kwon, Vattem, & Shetty, 2006; Tadera, Minami, Takamatsu, & Matsuoka, 2006). In T2DM, the post-prandial blood glucose level rises uncontrollably leading to hyperglycemic conditions causing metabolic derangements in the body.

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http://dx.doi.org/10.1016/j.foodchem.2016.05.090
0308-8146/

Treatment for hyperglycemia mainly focuses on stimulating insulin secretion from the β-cells of pancreatic islets, inhibiting the insulin degradation process, repairing or regenerating pancreatic β-cells, and inhibiting the starch hydrolases, α-amylase and α-glucosidase (Jarald, Joshi, & Jain, 2008). Therefore, α-amylase is one of the enzymes of great concern to the medical practitioners and researchers in controlling T2DM.

Compounds present in plant extracts are reported to act as inhibitors of α-amylase and several studies have been conducted to examine the anti-amylase activity of these plant extracts (Adisakwattana, Lerdsuwankij, Poputtachai, Minipun, & Suparpprom, 2011; de Sales et al., 2012; Mentreddy, 2007; Shori, 2015; Striegel, Kang, Pilkenton, Rychlik, & Apostolidis, 2015). Alpha amylase inhibitory property of compounds present in plant extracts may be determined by observing the changes in the physical property of substrates such as, (a) decrease of viscosity of a starch solution, (b) decrease of turbidity of a starch suspension, (c) decrease of intensity of a starch-iodine reaction and (d) increase of reducing power (Jarald et al., 2008). There are numerous methods used for the determination of amylase activity, of which 3,5-dinitrosalicylic acid (DNSA) assay is the most widely used. However, these methods share a common problem of being labor intensive, time consuming and require substantial amount of reagents and samples. In addition to this, there are many assay kits available in the market that measures amylase activity using a synthetic substrate such as ethylidene-pNP-G7, Benzylidene-G_7-α-PNP (Hu et al., 2014) and amylose azure (Klein & Foreman, 1980). Reliability of results obtained using synthetic substrates are been questioned in many papers due to lack of originality (Osman, 2002).

In the case of the enzyme amyloglucosidases, the glucose assay kit is used to measure enzyme activity by measuring the amount of glucose formed. The reaction taking place is,

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{POD(Peroxidase)}} \text{quinone} + 4H_2O$$

The glucose oxidase enzyme (GOD) used in these kits is a flavoprotein (Raba & Mottola, 1995) reported to be highly specific for glucose and according to reported literature there is no significant reaction with other sugars such as maltose, maltotriose and so on (Bankar, Bule, Singhal, & Ananthanarayan, 2009; Ferri, Kojima, & Sode, 2011; Schleis, 2007). In many studies, when their primary aim is to assess amylase activity, an enzyme such as amyloglucosidase or maltase is used to hydrolyze the product of amylase following the measurement using the glucose assay kit (Farias et al., 2010; Jeong, Harada, Yamada, Abe, & Kitamura, 2010; Proelss & Wright, 1975). However, from our preliminary attempts we observed that GOD is reacting with maltose which is the primary product of α-amylase action. Therefore, the present study aimed at developing an easy, simple and cost effective laboratory method to measure the α-amylase activity in a short period of time.

2. Materials and methods

2.1. Reagents and instruments

Glucose assay kit (GOD/POD) was purchased from Global Diagnostics and Medical Solutions, Belgium. Alpha amylase (A3306) and amyloglucosidase (A9913), soluble potato starch (33615), gallic acid (G7384), (+)-catechin hydrate (C1251), rutin hydrate (R5148), maltose monohydrate (M5895), dinitrosalicylic acid (609-99-4), potassium sodium tartrate tetrahydrate (6381-59-5), Sodium phosphate monobasic (71496) and sodium phosphate dibasic (71640) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Anhydrous dextrose (RM077) was purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). All the other chemicals used in the study were of analytical grade. Acarbose tablets available in the market were purchased for the study. The absorbance readings were taken using the Thermo Scientific Multiskan GO Microplate Reader.

2.2. Determination of lambda max (λ_{max}) and sensitivity of the assay

The maximum absorption wavelength was determined by running a spectrum from 400 nm to 700 nm. A 10 g/L starch solution was prepared in sodium phosphate buffer saline (PBS) (0.02 M, pH 6.9) by heating in a hot plate for 20 min at 100 °C and the freshly prepared starch solution was diluted to 2 g/L and 1 g/L. Maltose (5 mg/mL) and glucose (2 mg/mL) standard solutions and the enzyme α-amylase (2 U/mL) were prepared. The test was carried out in 96-well microtitre plates in a total volume of 260 μL. First, 80 μL of PBS and 40 μL of α-amylase were added. The reaction was started by adding 40 μL of substrate (Maltose/Glucose/Maltose + Starch/Glucose + Starch/Starch (1 g/L or 2 g/L)) into each well and the content was incubated for 15 min at 37 °C. Next, 100 μL of glucose kit reagent was added and was incubated for another 15 min and the absorbance was taken from 400 to 700 nm to select the best wavelength for the assay. Test blanks for substrates were done without starch and another with 2 g/L starch (without the enzyme) to detect any interference from the substrate to the assay.

2.3. Determination of linearity and sensitivity of the test

Linearity and sensitivity of the test method for maltose were tested against glucose. First, maltose and glucose standard series were prepared and the test was performed as mentioned in Section 2.2. The sensitivity of the test was assessed by determining the concentration required to give a minimum absorption of 0.1.

To estimate the sensitivity of the current method it was compared with the DNSA method. The DNSA assay was performed as described by Sudha, Zinjarde, Bhargava, and Kumar (2011), with modifications. The total assay mixture composed of 80 μL of PBS, 40 μL of α-amylase solution (2 U/mL) and 40 μL maltose, concentrations ranging from 0.1 to 5 mg/mL. The content was incubated at 37 °C for 15 min. The reaction was terminated with 100 μL DNSA reagent, placed in boiling water bath for 15 min, cooled to room temperature, diluted with 900 μL of deionized water and the absorbance was measured at 540 nm. The GOD method was done as described in Section 2.2.

2.4. Confirmation of detection of maltose

In to the wells, 40 μL of maltose, concentration ranging from 0.01 to 3 mg/mL, 80 μL of PBS and 40 μL of amyloglucosidase (AG) were added. Into another set of maltose standard series, instead of AG, 40 μL of PBS was added. After 15 min of incubation at 37 °C, 100 μL of glucose kit reagent was added and the absorbance was taken after 15 min at 500 and 505 nm for the set with the enzyme and without the enzyme respectively.

2.5. Determination of enzyme activity at different starch and enzyme concentrations

The optimum enzyme and substrate concentrations for the assay were determined using different concentrations of α-amylase and soluble starch. First, a stock of 20 U/mL of α-amylase and 10 g/L of soluble starch were prepared. The stocks

were diluted to appropriate levels to get enzyme concentrations of 1, 2, 3, 4 U/mL and starch concentrations of 0.5, 1, 2, 3, 4, and 5 g/L. Enzyme, starch, PBS and GOD reagent were added and the readings were taken immediately for 30 min in 1 min interval at 505 nm.

2.6. Application of the GOD method

2.6.1. Sample preparation

Acarbose was prepared in PBS and the phenolic acids, catechin, gallic acid and rutin stock solutions were prepared and were diluted with PBS. The concentration series used for the study were acarbose (2, 4, 6, 8, 10, 20 μg/mL), gallic acid (2.5, 5, 10, 15, 20, 25, 50 μg/mL), catechin and rutin (7.5, 15, 150, 300 μg/mL).

2.6.2. Procedure

The assay system comprised the following components in a total volume of 260 μL: 40 μL of PBS (0.02 M, pH 6.9), 100 μL of GOD reagent, 40 μL of each, soluble starch (2 g/L), inhibitor solution and the enzyme solution (2 U/mL). Briefly, the enzyme was mixed with the inhibitor solution and pre-incubated on a hotplate for 10 min at 37 °C. The reaction was started by pipetting the starch solution into the wells and was incubated for another 15 min. Finally, 100 μL of the GOD reagent was added and the absorbance was measured at 505 nm after 15 min. As negative control, instead of sample, 40 μL of PBS was used and the absorbance was measured parallel with samples. For each concentration of the sample, a sample blank was added in parallel. For kinetic studies, immediately after addition of starch, GOD reagent was added and the readings were taken at 1 min interval for 45 min at 505 nm. The results were expressed in terms of IC_{50} value.

2.7. Determination of precision and reproducibility

The precision of the test was done using acarbose as the inhibitor. The precision was tested by testing the intra-day and inter-day variability for the same test. The test was performed as mentioned in Section 2.6 with three replicates. The same experiment was continued for 5 days under same experimental conditions. The intra-day precision of the test was determined by comparing the IC_{50} values of the replicates and the inter-day precision/reproducibility by comparing the IC_{50} values obtained for 5 days.

2.8. Comparison between DNSA and GOD method

A comparison was done between the DNSA and GOD method using acarbose. The test for DNSA and GOD was performed as mentioned in Section 2.3 and 2.6 respectively. In the DNSA method, instead of 80 μL of PBS 40 μL was used. Next, 40 μL of acarbose was incubated with the enzyme for 10 min and the assay was continued as mentioned in Section 2.3. The GOD method was done as mentioned in Section 2.6 and the IC_{50} values were calculated.

2.9. Statistical analysis

Data were analyzed using the SAS statistical software version 9.1.3 (SAS Institute Inc., Cary, NC). Results were calculated and expressed as mean ± standard deviation (SD) of 3 independent analyses. P values of < 0.05 were considered to be significant. Graphical presentation of data was done using the GraphPad prism 5 software.

3. Results and discussion

3.1. Determination of lambda max and sensitivity of the assay

First-of-all, the optimum wavelength where maximum absorption takes place was determined by running a spectrum in the visible range between the wavelengths of 400–700 nm. Maltose showed maximum absorbance at a wavelength of 505 nm whilst the glucose standard showed maximum absorbance at 500 nm (See Fig. S1 in Supplementary material). However, there was no significant difference ($P > 0.05$) between the absorbance values at 500 and 505 nm for maltose or glucose. The deviation was around 0.0016 and 0.0003 for maltose and glucose respectively. Anyhow, the results showed that the absorbance measured at 505 nm gave the lowest detection limit indicating highest sensitivity of measurement at this wavelength. Therefore, the wavelength of 505 nm was chosen for the study.

As can be seen from Fig. S1 (Supplementary material), there was no interference from the substrate starch at the tested wavelengths. That is, the presence of starch, the main substrate in the α-amylase assay, does not change the spectral properties of the final product which makes the new method more precise and less complex.

3.2. Determination of linearity and sensitivity of the test

Validation of the new method was carried out as described in the ICH Harmonized Tripartite Guidelines (ICH, 2005). Linearity of an analytical method is the ability of the method to generate results directly proportional to the concentration of the analyte present in the samples, in a known range of concentrations (Negrulescu et al., 2012). As can be seen from Fig. 1a and b, the calibration curves obtained from the new method for both glucose and maltose were all linear with a correlation of determination (R^2) value above 0.99. The high values obtained for R^2 suggest a strong linear relationship between the two variables tested. To further confirm and to eliminate the doubt of specificity of the kit used, the test was done with two other glucose assay kits available in the market (LS 350 and A166, Biolab Diagnostics, Pvt. Ltd). The test results confirmed the interaction of GOD with maltose.

Sensitivity of the glucose kit for maltose was done by drawing a maltose standard curve where the extinction coefficient values obey with the Beer Lambert's law. For the same concentration, the absorbance measurement for glucose was much higher than maltose and it did not fit in the linear range. Therefore, separate concentration series were prepared for maltose and glucose (Fig. 1). The lowest detection limit for the sugars was set as the lowest concentration required to get an optical density (OD) of 0.1 and a R^2 value above 0.99. The lowest detection limit for maltose and glucose within the linear range was 0.25 mg/mL (OD = 0.1, $R^2 = 0.99$) and 0.01 mg/mL (OD = 0.10, $R^2 = 0.99$) respectively. The sensitivity of maltose was approximately 25 times lower than glucose (Fig. 1a, b). GOD is highly selective toward β-D-glucose, and the enzyme shows considerably reduced activity with other sugars such as maltose. The relative oxidation rate of maltose by GOD is reported to be only 0.19 when the rate for β-D-glucose is set at 100 (Raba & Mottola, 1995). Therefore, lower sensitivity of maltose may be due to the reduced affinity of GOD for maltose.

DNSA method is the most widely used assay for testing anti-amylase activity (Gonçalves, Rodriguez-Jasso, Gomes, Teixeira, & Belo, 2010; King, Donnelly, Bergstrom, Walker, & Gibson, 2009; Negrulescu et al., 2012). The DNSA, as well as the newly developed GOD method, both measure the amount of reducing sugar produced. Therefore, we compared the sensitivity of both the assays for maltose. As can be seen from Fig. 1c, at a maltose concentration

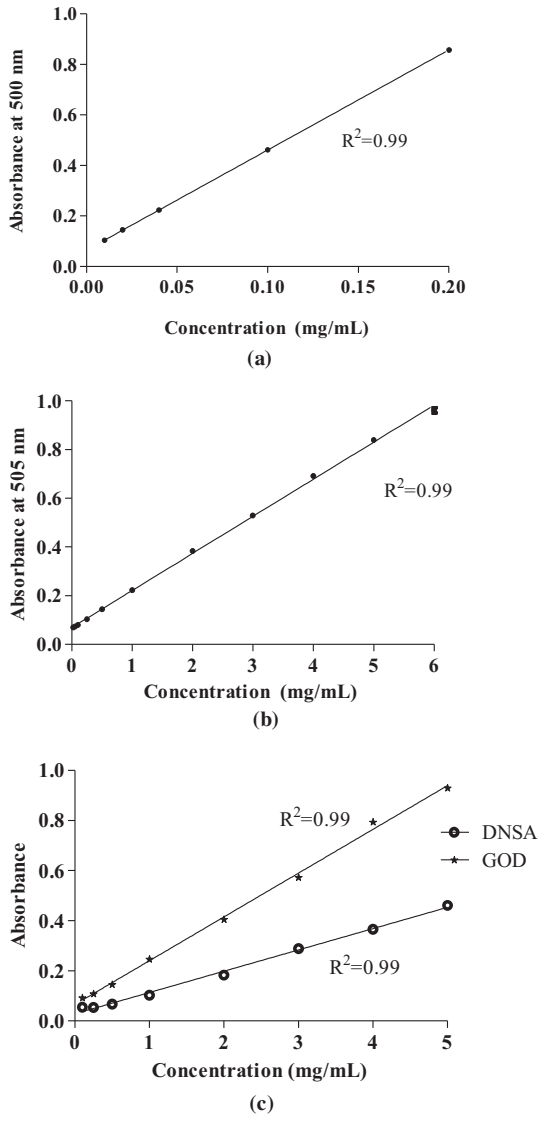

Fig. 1. Standard curve for (a) glucose and (b) maltose for the GOD method and (c) comparison of the DNSA and GOD method for sensitivity for maltose.

of 5 mg/mL, the absorbance reading for the GOD and DNSA method was 0.93 and 0.46 respectively. When comparing the slopes of the standard curves obtained, a decrease in sensitivity is clearly shown by the decrease of the slope from 0.175 ± 0.003 (GOD) to 0.085 ± 0.002 (DNSA). The new method showed higher sensitivity for maltose than the DNSA method, where the lowest detection limit for maltose in the DNSA method was 1 mg/mL (OD = 0.1) while in the GOD method it was 0.25 mg/mL (OD = 0.1). Considering the lowest detection limit, sensitivity for maltose was roughly 4-fold higher than the DNSA method (Fig. 1c) which makes the new method more precise and sensitive for the determination of anti-amylase activity of plant extracts.

3.3. Confirmation of detection of maltose

The glucose assay kit is designed specifically for the detection of glucose in serum samples. According to literature, the kit is specific for glucose and there is no interference from maltose or other reducing sugars (Bankar et al., 2009; Ferri et al., 2011; Schleis, 2007). Thus, to further confirm that the kit reagent is reacting with maltose, we compared the OD values of the maltose curve with and without the enzyme amyloglucosidase. Amyloglucosidase hydrolyzes maltose to its respective monomer glucose (Toma, Makonnen, Mekonnen, Debella, & Addisakwattana, 2014). Since the maltose standard used is of high purity (>98%), it eliminates the possibility of presence of glucose which might give rise to false positive results. As discussed above, the sensitivity of the GOD kit reagent for glucose is higher than that of maltose. As expected, the absorbance values of the maltose standards treated with the enzyme amyloglucosidase were higher than the untreated maltose standards (Fig. 2a). This confirms that the colored complex or the red quinone formed is as a result of the interaction of GOD with maltose. In addition, a positive correlation (Fig. 2b) between the OD of maltose and glucose (produced from maltose through AG action) further confirms that maltose reacts with GOD.

3.4. Determination of enzyme activity at different concentrations

To find the optimum enzyme concentration for the assay, enzyme concentrations of 1–4 U/mL were prepared and the absorbance was measured at 505 nm for 30 min (Substrate concentration 2 g/L). Increase in enzyme concentration significantly increased the absorbance reading indicating increased enzyme activity (See Fig. S2(a) in Supplementary material). Absorbance values for the concentrations of 2, 3 and 4 U/mL were within the acceptable range, that is, after 30 min, the OD was between 0.25 and 0.4. But in the case of 1 U/mL, the OD was below 0.20.

When the substrate concentration was increased, the absorbance value also increased proportionally, indicating enhanced enzyme activity (See Fig. S2(b) in Supplementary material). For the substrate concentrations 0.5 and 1 g/L, the OD values were below 0.2 and in case of 4 and 5 g/L the reaction rate was too rapid. According to the results, substrate concentrations 2 and 3 g/L are appropriate for the assay. Therefore, further tests were carried out using the enzyme concentration of 2 U/mL and substrate concentration of 2 g/L. Henry and Chiamori (1960), studied the hydrolysis of various starch preparations by α-amylase in detail and reported a lag-phase of about 15 min for the formation of maltose and maltotriose. Therefore, in our study, we incubated the substrate with the enzyme for 15 min to allow enough time for the reaction to take place followed by the addition of glucose kit reagent. It was incubated for an additional 15 min to allow enough time for color development.

3.5. Application of the GOD method

The study on amylase inhibitory activity of plant compounds is a widely studied area which may be relevant to the treatment of T2DM (Jarald et al., 2008; Mentreddy, 2007; Patel, Prasad, Kumar, & Hemalatha, 2012). To-date, about 800 plant species have been reported to exert anti-diabetic activity (de Sales et al., 2012). Compounds such as glycosides, flavonoids, carotenoids, galactomannan gum, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides and terpenoids are reported to be responsible for the exerted bioactivity against hyperglycemia (Mentreddy, 2007; Patel et al., 2012).

Acarbose is a well-known inhibitor of α-amylase and α-glucosidase which is prescribed as a drug to treat hyperglycemic conditions (Robyt, 2005) and is also being used as a positive standard in assessing α-amylase and α-glucosidase inhibitory effect of plant extracts (Adisakwattana et al., 2011; Liu et al., 2011). Therefore, in our study, acarbose was used as the reference standard to assess α-amylase inhibitory effect of some phenolic acids such as catechin, gallic acid and rutin.

It can be seen from Fig. 3 that the activity of the enzyme is retarded with increasing concentrations of acarbose and phenolics. It is evident from the results that none of the phenolics tested were

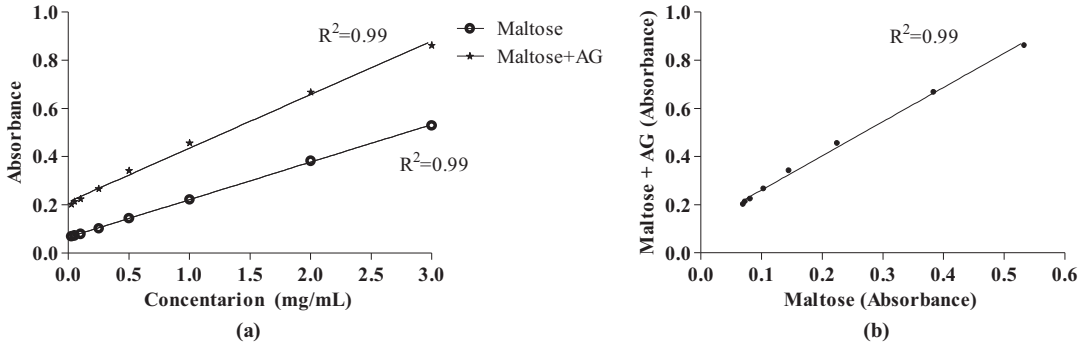

Fig. 2. (a) Comparison of Amyloglucosidase (AG) treated and untreated maltose, (b). Correlation between treated and untreated maltose.

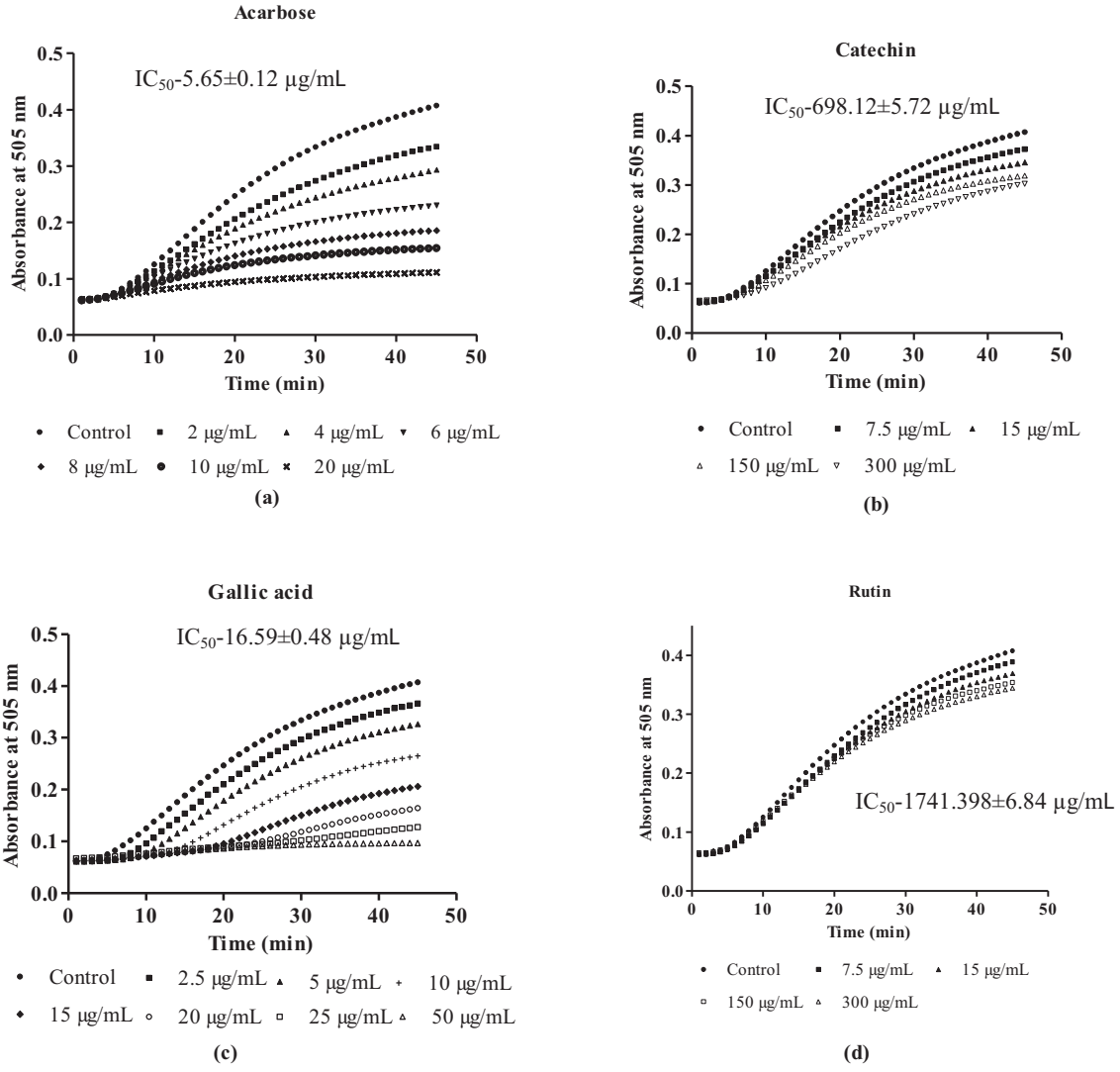

Fig. 3. Representative kinetic curves for α-amylase activity in the presence of inhibitors. Color development at different concentrations of (a) acarbose, (b) catechin, (c) gallic acid and (d) rutin.

effective as acarbose in inhibiting the activity of α-amylase. As compared to the DNSA method, it should be noted that the new method is more convenient in doing kinetic studies, where the reagents and plant extracts are put into the microtitre plate and the readings taken at once, which is less time consuming and less laborious.

3.6. Determination of precision and reproducibility

The precision of the test was determined by testing the repeatability of the results obtained by running the acarbose standard for 5 days in triplicates. According to the data presented in Table 1, it is evident that the method has excellent precision. The intra-day and

Table 1
Accuracy and precision of the GOD method.

Day	Acarbose IC_{50} (μg/mL)			Mean ± SD	% Precision
	R1	R2	R3		
1	5.13	5.15	4.93	5.07 ± 0.12	97.6
2	5.27	5.24	5.05	5.19 ± 0.12	97.7
3	4.98	4.93	4.95	4.95 ± 0.03	99.4
4	5.13	5.19	5.09	5.14 ± 0.05	99.0
5	5.00	4.94	4.98	4.97 ± 0.03	99.4
Mean				5.06 ± 0.10	

inter-day relative standard deviations were 1.38% and 1.98% respectively. The outcome of the test demonstrate that the results are highly reproducible. The precision of the test can be predicted by looking at the replicate values, where the standard deviation in all occasion is less than 0.2 and the percentage precision of the test for all five days was above 97%. In addition, the readings taken for 5 days reveals high reproducibility for the acarbose test where the mean IC_{50} value for acarbose for all 5 days is 5.06 μM with a SD value of 0.1 and the results were not significantly different ($P > 0.05$) as analyzed by the completely randomized design (CRD) test.

3.7. Comparison of DNSA and GOD method in assessing inhibition

Inhibition of α-amylase by acarbose was quantitated using the DNSA and the GOD method. The linearity/correlation of determination for varying concentrations of acarbose was greater for the GOD method than the DNSA method (Fig. 4a). According to the results, the IC_{50} values for acarbose by the GOD and DNSA method were 5.51 ± 0.14 μg/mL and 17.86 ± 0.24 μg/mL respectively. There is approximately three fold difference in the values obtained from both the methods. For the same compound, different methods are reported to give different values (Liu et al., 2011; Tadera et al., 2006; Wang, Liu, & Huang, 2013). For example, in a study done by Manaharan, Appleton, Cheng, and Palanisamy (2012), quercetin was reported to show comparable activity as that of acarbose by the DNSA method while, based on turbidity measurements it was found to be inactive (Wang et al., 2013). In another study, luteolin which was reported to be a potent inhibitor of amylase (Kim, Kwon, & Son, 2000) was found to exhibit no measurable activity using the turbidity method (Liu et al., 2011). The exact reason for differences in results is still not known and requires further investigation. However, a good correlation of determination ($R^2 = 0.9973$) between the two methods (Fig. 4b) indicate that both the methods have almost identical responses to varying acarbose levels. This rules out the doubt of the applicability of the new method in assessing the α-amylase inhibitory activity.

Compared to the DNSA method, the GOD method is labor saving and requires less time. The major disadvantage of the DNSA assay is the heating step that is required for color development (Gonçalves et al., 2010; Negrulescu et al., 2012). In the DNSA method, after treating the enzyme with plant extract and starch, the content is heated at 90 °C for 15 min. Then it is cooled and diluted with distilled water and the absorbance is measured at 540 nm. On the other hand, in the GOD method, after treating the enzyme with the plant extract, starch and the GOD reagent can be added at once and the absorbance reading can be taken after 30 min or for kinetic studies, the absorbance readings can be taken from that point itself.

4. Conclusions

The adapted method is quick, reproducible, and less laborious and the materials required for the assay are relatively inexpensive and are easily available. In the present work, the developed GOD method was successfully used to determine the α-amylase inhibitory activity of acarbose and three phenolics. The results demonstrate that the developed method is accurate, quantitative, precise and highly reproducible. The new method can be considered as a high-throughput technique as it allows the analysis of several samples at once and also it is most suitable for kinetic studies.

Acknowledgements

The authors are thankful to the National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka for providing financial and analytical support and also would like to convey our special thanks to Professor B.M.R. Bandara, Department of Chemistry, Faculty of Science, University of Peradeniya, Sri Lanka for his valuable comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.05.090.

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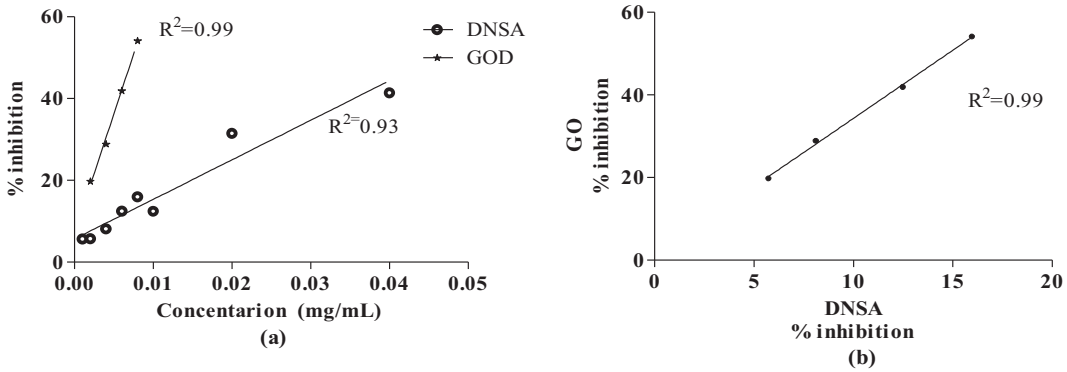

Fig. 4. Comparison between GOD and DNSA method (a). Acarbose inhibition curve against concentration, (b). Correlation between GOD and DNSA method.

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